CHROMATOGRAPHY OF THE FREE AMINO ACIDS IN BLOOD PLASMA OF CHICKENS INFECTED WITH CECAL COCIDIOSIS

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ABSTRACT

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Chromatography of Free Amino Acids in Blood Plasma of Chicks Infected with Cecal Coccidiosis

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A chromatographic analysis of the free amino acids in blood plasma of chicks infected with cecal coccidiosis was made. The investigation was conducted for a period of 120 days.

Blood was obtained by cardiac puncture and the plasma prepared for chromatography with a chemical technique using hydrochloric acid, acetone and ether. With this technique, the proteins were precipitated and both lipids and organic salts were removed. The amino acids were identified by running known amino acids simultaneously with the plasma samples and subsequently calculating their R_f values. Descending chromatography was employed and the color developed with 0.25% ninhydrin in acetone.

Quantitative determinations were made using a spot area—concentration method. The area was determined by outlining the spots on graph paper and counting the squares.

The concentration of the amino acids was apparently reduced during the height of the infection.

ACKNOWLEDGEMENTS

I wish to thank Dr. George Riley and Dr. Barnett Smith for helpful suggestions and encouragement. Without their help this investigation would never have been completed.

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CHAPTER I

INTRODUCTION

Since Eimer's observation in 1870 of the coccidian parasite in the intestine of fowl, much work has been done in an attempt to outline and categorize the physiological aspects of cecal coccidiosis infections in chickens. The symptoms of the infection show physiological upsets which range from an increase in temperature to pronounced hemorrhage and death. As a result of this hemorrhage which is associated with the infection, numerous studies have been made on the blood of infected chicks. In spite of the fact that blood sugars, blood chlorides, blood proteins and many other blood substances have been studied with respect to cecal coccidiosis, the free amino acids of the blood have, seemingly, escaped investigation.

Amino acids are the structural units of proteins and may be regarded as derivatives of the saturated fatty acids series containing at least one carboxyl (COOH) and one amino (NH_2) group. When an amino acid is uncombined it is referred to as a free amino acid. The blood plasma contains many free amino acids and paper chromatography has provided an excellent method for their identification as well as for an estimation of their concentration.

The need for additional physiological information on the host-parasite relationship in cecal coccidiosis is indicated by the pronounced inadequacy of any present theory to explain certain physiological mani-

festations of the infection.

This investigation was made to determine the effects, if any, that cecal coccidiosis infection has upon the free amino acid content in blood plasma of chickens.

CHAPTER II

REVIEW OF LITERATURE

While it seems certain that different authors have observed separation by filtration through columns of finely divided adsorbents, the Russian botanist Tswett, was the first to be aware of the great possibilities of chromatography (Lederer and Lederer, 1957). He applied the name chromatography to the separation of xanthophylls, chlorophylls and carotenes by filtration through columns which was followed by color development using pure solvents. His first paper, published in 1903, contains a study of more than 100 adsorbents used in conjunction with several different solvents and a comparison of the efficiency of column adsorption. Calcium carbonate firmly packed in a glass tube was a favorite adsorbent while petroleum ether was a frequently used solvent. The column with the separated pigments was called a chromatogram and the entire process the chromatographic method.

There are many different kinds of chromatography (flowing, gas-liquid, ion-exchange, paper, column, etc.) but since the now classical work of Condsen, Gordon, and Martin (1944), the use of the paper chromatographic technique has become very popular in many areas of biochemical research. These investigators considered filter paper as an inert support of an aqueous stationary phase and explained the observed separations as a result of continuous partitions of the substances between the aqueous phase and the water immiscible organic solvent flowing down the paper.

Thus, paper chromatography is a type of partition chromatography distinct from the adsorption chromatography of Tswett. Although other names such as papyrography and partography, have been proposed for the process, the name paper chromatography has somehow seemed to persist.

Dent (1947) stated that while paper chromatography may be applied to many other types of compounds, it seems likely that its greatest value will continue to be in the amino acid field. Dent studied the behavior of some 60 amino acids and noted the occurrence of some of them in biological fluids. Some of the amino acids often found in blood, urine and other biological fluids are lysine, alanine, isoleucine, leucine, glycine, glutamic acid, valine, threonine and histidine.

Walker (1952), using the paper chromatographic technique, studied the free amino acids occurring in the tissue fluids and blood of rats and cows. Fresh blood was withdrawn from the aorta of the rat and cow with heparin used in the receiving vessel to prevent clotting. The blood was centrifuged as soon as possible after the collection. The plasma was pipetted off and the cells hemolysed with an equal amount of water. Proteins in the plasma and hemolysed cells were precipitated in several different ways. Plasma precipitation was effected by treating 1 ml. of plasma with 10 ml. of 95% ethanol and, after allowing the mixture to stand for 15 minutes, the precipitate was obtained after centrifugation. The ethanol was removed from the supernatant by evaporation in a current of warm air. The residue was taken up in a known volume of water, desalted in an electrolytic desalting apparatus designed by Consden, Gordon and Martin (1947), and quantities equivalent to 250 microliters of blood cells and plasma were applied to chromatograms. Ultrafiltration and tungstic acid precipitation were also tried. Although

protein precipitation using ethanol produced the clearest chromatograms, losses of amino acids apparently occurred and glutathione especially was in smaller concentration when compared with results obtained by ultra-filtration. Aspartic acid was always present but in small amounts.

Gordon and Nardi (1954) modified a chemical method of two French investigators (Boulanger and Biserte, 1949) and were able to produce satisfactory chromatograms of human blood plasma. They noted that there were numerous technical difficulties involved in a paper chromatographic analysis of blood. Blood plasma contains three substances primarily responsible for the difficulties. These are salts, proteins, and lipid materials. Consden, et. al. (1947) devised an electrolytic desalting machine to remove the salts but it has been shown by Stein and Moore (1951) that there is a loss of some of the amino acids during this process. In addition, the method is very cumbersome. They also believed that although the proteins can be precipitated by the usual protein-precipitating agents and by ultra-filtration, the former also resulted in amino acid losses and the latter was much too tedious. Probably the most efficient way to achieve separation of the amino acids would be by a series of ion-exchange resins as illustrated by the work of Moore and Stein (1951). This technique, however, is extremely tedious and time consuming and does not lend itself to a study of samples of small volume and concentration. The presence of lipid material resulted in a technically unsatisfactory chromatogram with smearing and smudging.

Gordon and Nardi extracted the amino acids from the dried plasma of human blood with a solution containing 1.6 ml. of 6N HCL per 100 ml. of acetone, in which the inorganic salts are insoluble. Lipids were removed from the extract with ether. The remaining residue was then dried

in a vacuum desicator over sulfuric acid and evaporated to dryness. The dried residue was then dissolved in 25-100 micro-liters of water and applied to the paper for chromatography.

Two-dimensional chromatography was usually carried out using phenol and water as the first solvent and butanol, propionic acid and water as the second solvent. The dried two-dimensional chromatograms were developed with an ethanolic solution of 0.1% ninhydrin and the color allowed to develop overnight at room temperature. Both plasma and serum were analyzed and the amino acids identified were aspartic acid, cysteine, serine, glutamic acid, glycine, threonine, alanine, valine, isoleucine, and proline. Identification of the spots was made by adding a pure amino acid to a sample before chromatographic analysis and verifying the re-enforcement in color of a particular spot and failure of a new spot to appear. In addition to the 10 amino acids identified, 6 other spots were unidentified.

Because of its simplicity as a qualitative technique, many investigators have attempted to develop paper chromatography into a quantitative or semi-quantitative technique. These techniques are based essentially on (1) matching colors on the paper (Polson et al. 1947), (2) extraction of the amino acids from the chromatograms and subsequent determination by conventional micro-methods (Woiwod 1949), (3) determination of the area occupied by substance on the paper and R_f values (Fisher, Parson and Morrison 1948), and (4) photoelectric densitometry of the colored substances (Rockland and Dunn, 1949).

Fisher et al. (1948) developed a method of quantitative assay of amino acids based on the spot area-concentration relationship. Not only did they find that the size of the spots diminishes regularly with decreas-

ing concentration, but also there is a slight variation in the R_f value with a change in concentration. They used a mixture of alanine, glutamic acid and glycine, each of which had a concentration of 0.062 M. Several dilutions were made with each having 2/3 the concentration of the previous one (dilution factor = 2/3). The outlines of the spots were made sharp with a commercial reflex copier (Miles Aircraft Company "Copycat"), and the area of the spots were measured with a planimeter. They found that the areas were linearly related to the logarithm of the amount of amino acid present.

In 1939, Herrick found that "with the onset of hemorrhage, there was a reduction of erythrocytes, blood volume and hemoglobin, a depletion of liver and muscle glycogen, a decrease in total and non-protein nitrogen and a reduced metabolic rate. Concomitant with these changes, there was an increase in the sugar and sodium chloride content in the blood with the osmotic pressure remaining relatively unchanged." Herrick's observation that both total and non-protein nitrogen was reduced provided the basis for this investigation.

By artificially bleeding the chicken, Pratt (1940 and Waxler (1941) reported an increase in blood sugars and blood chlorides, respectively. They then drew the much questioned conclusion that the hemorrhage itself was the primary cause of the physiological upset rather than the infection of the parasite.

Schlueter (1959), using the microelectrophroretic technique, reported a drop in the relative albumin and globulin level in serum on the fifth day after infection with coccidia.

CHAPTER III

MATERIALS AND METHODS

White Leghorn cockerels were obtained from the To-Lani Farm of Stone Mountain, Georgia. They were incubated and grown at the farm under conditions as nearly coccidian free as possible. In our laboratories, the chicks were separated into two groups, kept in wire cages and fed on Purina growing mash. One group was used as controls and the other designated as the experimentals. Care was taken to prevent accidental infection of the controls.

The sporulated oocysts were obtained from Dr. Challey of the University of Wisconsin and propogated in the laboratories of Spelman College, Atlanta, Georgia. Small doses were given several chicks and the unsporulated oocysts recovered from the cecal pouches on the ninth day after the infection. These unsporulated oocysts were placed in Petri dishes containing a 2.5% solution of potassium dichromate and allowed to sporulate in air. With the use of a hemocytometer, 150,000 to 200,000 oocysts were counted and given orally to several chicks with a pipette. Each infected chick was placed in a wire cage and observed daily.

Obtaining the blood:

Blood for analysis was obtained from uninfected control chicks, as well as from chicks infected with Eimeria tenella on the fifth to ninth day after infection. The small feathers were plucked from the cardiac region and the region sterilized with alcohol preparatory to obtaining blood by cardiac puncture. Five milliliter syringes and No. 22

guage needles were used to obtain blood. Centrifuge tubes were prepared to prevent clotting using a double oxalate technique. With this technique, 1.2 gm. of ammonium oxalate and 0.8 gm. of potassium oxalate were dissolved in 100 ml. of water. One-tenth milliliter of the double oxalate for each milliliter of the blood to be collected was placed in each syringe and centrifuge tube. They were then dried in an incubator overnight at 80° C. Before use, they were chilled for 12 hours or more in an ice bath or freezer. When the blood was drawn, it was centrifuged immediately to prevent hemolysis. If hemolysis or injury to the blood cells occurred, the amino acids of the cells would diffuse into the plasma. This would cause an error in the analysis of the plasma.

Plasma, rather than serum, was analyzed because of the desire to determine the amino acid content of the circulating plasma. Mac Fadyen (1942), observed that when blood coagulates, amino acids were set free by reactions not yet fully understood, so that the alpha amino nitrogen of serum may be 20% higher than that of plasma. Hence, the anti-coagulant (double oxalate) was necessary. From 3 to 4 ml. of blood were obtained from each of several chicks and centrifuged at 2000 r.p.m. for 15 minutes. The plasma obtained was used immediately.

Preparing the plasma for chromatography:

One milliliter of blood plasma was placed in a homogenizing tube, put in a vacuum dessicator over sulfuric acid and dried for 24 to 36 hours. The dried plasma was then removed and to it 8 ml. of an acetone solution containing 1.6 ml. of 6N HCl per 100 ml. of acetone was added. The dried plasma was then ground very finely, stoppered and agitated vigorously for 15 or 20 minutes. The acetone solution was then transferred to a 15 ml. centrifuge tube with as much of the precipitated proteins being

transferred as possible. Five milliliters of the acetone-hydrocloric acid mixture was then added to the residue in the homogenizing tube and agitated for 10 minutes. The transferred solution was centrifuged for 10 minutes and the supernatant removed and placed in a test tube. The second batch of the acetone solution in the homogenizing tube was transferred to the original centrifuge tube, centrifuged for 10 minutes and the supernatant removed and placed in the first supernatant. At the end of the process, the precipitated proteins were washed twice with the acetone-hydrochloric acid mixture and the washing collected in a single test tube.

The washing was then put into a small vial and placed in a 37° C. water bath. A stream of air, dried by passing it through a calcium chloride tube, was used to evaporate the acetone mixture until a yellow-brown residue was left in the vial. This residue contained the amino acids that were free of proteins and salts but contained lipids. Five-tenths of a milliliter of distilled water was placed in the test tube and the yellow-brown residue dissolved. An equal quantity of diethylether was added and the tube vigorously agitated for several minutes. The ether layer was removed with a micropipette and the ether extraction procedure repeated. This ether extraction procedure removed the lipid materials.

The remaining solution was again placed in the vacuum dessicator over sulfuric acid and evaporated to dryness. The dried residue was then dissolved in 100 to 150 microliters of water and the desired aliquots applied to the filter paper for chromatography.

One-dimensional chromatograms were made, dried, run again in the same direction and in the same solvent. Whatman No. 1 chromatographic

paper (46 x 57 cm.) was used for descending chromatographic separation. Chromatograms of known amino acids with known concentrations were run simultaneously with chromatograms of experimental samplas on the same piece of filter paper. Micropipettes were used to apply very small and equal volumes of known amino acids and plasma extracts to the paper 3 cm. apart and 50 cm. fron the end of the paper. After the chromatograms had run for 11 hours in a chamber (70 x 70 x 50 cm.) designed especially for descending chromatography, they were dried in air, placed back into the chamber and run again 11 hours. The solvent used was 1-butanol, acetic acid and water (4:1:4). For color development, the chromatograms were sprayed with 0.25% ninhydrin in acetone and allowed to develop in air. Identification of the amino acids was accomplished by calculating the rate of movement (R_f value) of the amino acids and comparing them with the spots of the sample.

A semi-quantitative technique was employed comparing the area of the spots with the concentration. After the spots had been identified, solutions of each amino acid were made containing 5 mg./100 ml. of water. Then 4 mixtures of alanine, glycine and ornithine were prepared with each containing 5, 3.75, 2.5, and 1.25 mg./100 ml. of water of these three amino acids. After similar chromatograms were obtained with these 4 mixtures the area of each spot was outlined with a sharp pencil and a carbon copy traced on graph paper. The area (number of squares) of each spot was counted and plotted on the graph paper against the concentration. In this way, the concentration of the spot could be determined by comparing the area of the spot with the area of its corresponding amino acid.

CHAPTER IV

EXPERIMENTAL RESULTS

A paper chromatographic analysis was made of the free amino acids in the blood plasma of normal chicks and of chicks infected with the coccidian parasite, Eimeria tenella. The investigation lasted for a period of 120 days.

Multiple developed chromatograms of normal and infected plasma revealed 9 ninhydrin positive substances. Of these 9 ninhydrin positive substances, 7 were identified as amino acids and 2 were unidentified. A list of these amino acids, together with their corresponding R_f values of normal and infected plasma is shown in Table I and Table II. Identification was made by running various known amino acids of previously determined concentrations simultaneously with the plasma samples and subsequently calculating the R_f values (Fig. 1). With the exception of tyrosine and spots in positions #4 and #8 (both unidentified), the sizes of the spots were fairly large and the colors moderately intensified. This would indicate a relatively high level of concentration. Glutamic acid and valine had the highest concentration and tyrosine had the lowest. A list of the free amino acids and their estimated concentration found in normal and infected plasma is given in Table III.

In the experimental chicks, there was a slight decrease in the size and intensity of the spots on the chromatograms. This would indicate a relative decrease in the amino acid concentration. Figures 2 and 3 show a chromatogram of both normal and infected plasma.

TABLE I

THE R_f VALUES AND CORRESPONDING FREE AMINO ACIDS

IN BLOOD PLASMA OF NORMAL CHICKS

Position on Chromatogram	Free Amino Acids in Plasma	R_f Values (22 hrs. at 30° C)* Experimental**	Theoretical***
1	Lyaine	.12	.11
2	Aspartic acid	.25	.25
3	Glutamic acid	.33	.33
4	Unidentified	.39	.39
5	Alanine	.45	.46
6	Tyrosine	.52	.52
7	Valine	.67	.66
8	Unidentified	.74	.74
9	Iso-leucine	.77	.77

$$R_f \text{ value} = \frac{\text{distance traveled by the amino acid}}{\text{distance traveled by the solvent fronts}}$$

*22 hrs. = these are multiple developed chromatograms run 11 hours in one direction, allowed to dry and run 11 more hours in the same direction.

**Experimental R_f = R_f value obtained after the solvent has migrated twice down the chromatographic paper; 11 hours each time at 30° C.

***Theoretical R_f = R_f value expected if the solvent migrates twice down the chromatographic paper; 11 hours each time at 30° C.

TABLE II

THE R_f VALUES AND CORRESPONDING FREE AMINO ACIDS IN BLOOD PLASMA OF CHICKS INFECTED WITH THE PARASITE, EIMERIA TENELLA

Position on Chromatogram	Free Amino Acids in Plasma	R_f Values (22 hrs at 30° C)* Experimental**	Theoretical***
1	Lysine	.12	.11
2	Aspartic acid	.24	.25
3	Glutamic acid	.33	.33
4	Unidentified	.27	.39
5	Alanine	.45	.46
6	Tyrosine	.52	.52
7	Valine	.67	.66
8	Unidentified	.74	.74
9	Iso-leucine	.77	.77

R_f value = $\frac{\text{distance traveled by the amino acid}}{\text{distance traveled by the solvent front}}$

*22 hrs. = these are multiple developed chromatograms run 11 hours in one direction, allowed to dry and run 11 more hours in the same direction.

**Experimental R_f = R_f value obtained after the solvent has migrated twice down the chromatographic paper; 11 hours each time at 30° C.

***Theoretical R_f = R_f value expected if the solvent migrates twice down the chromatographic paper; 11 hours each time at 30° C.

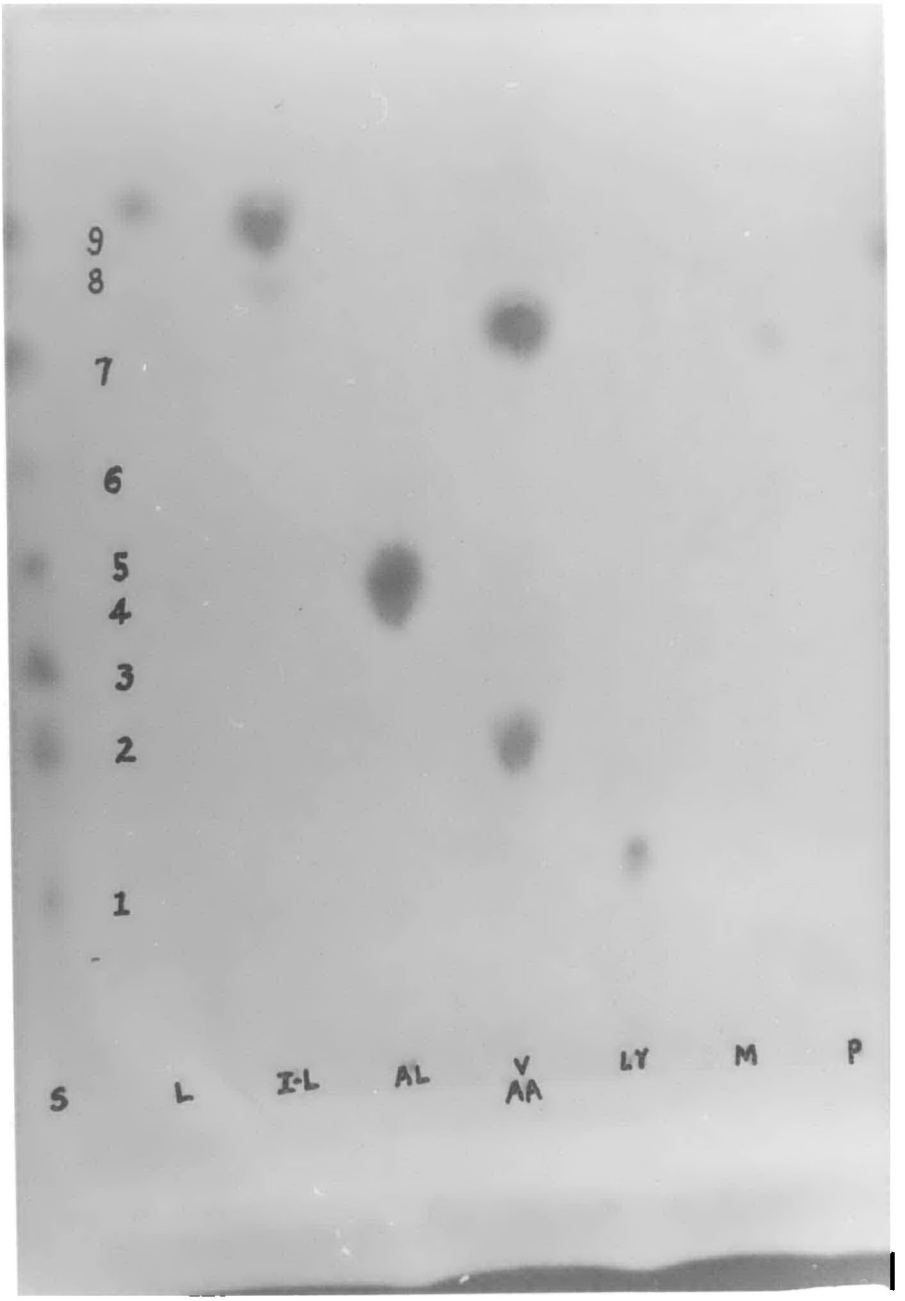


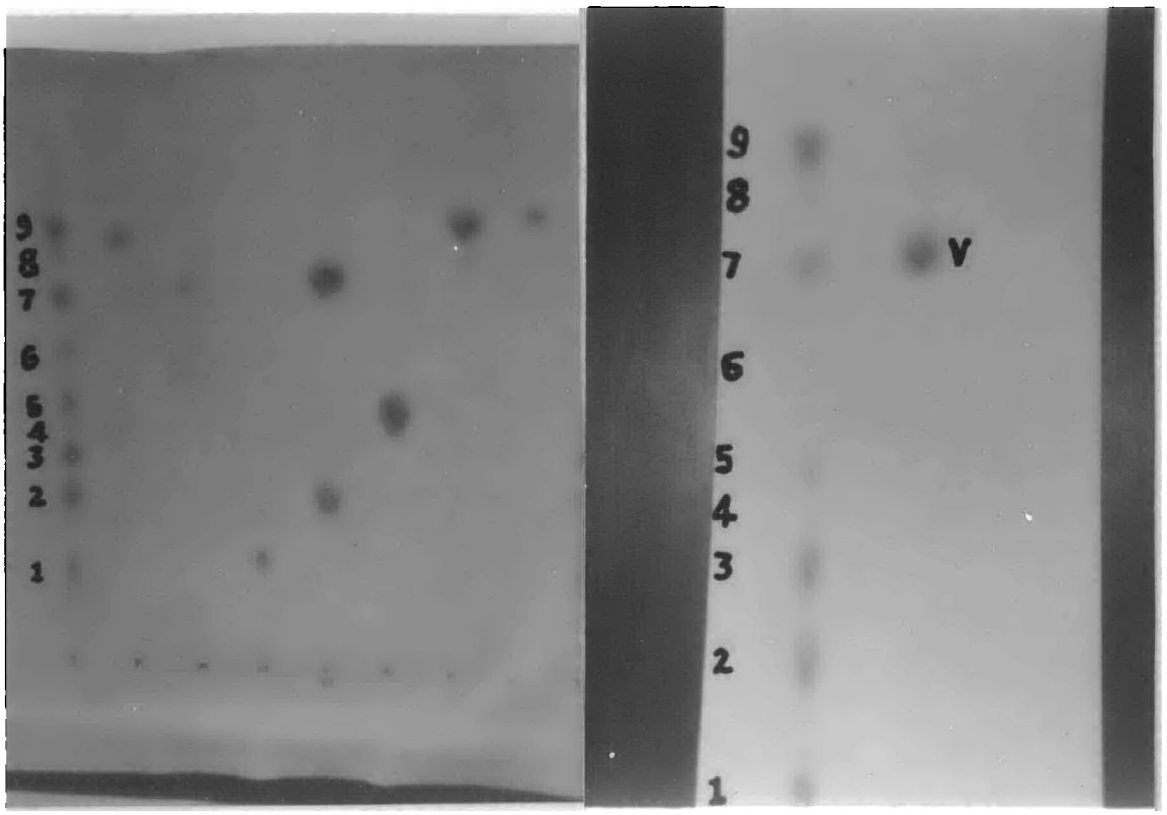
Fig. 1. A chromatogram of blood plasma of normal chicks showing known amino acids that were run simultaneously with the plasma sample. 1 and LY = Lysine, 2 and AA = Aspartic acid. 3 = Glutamic acid. 4 = Unidentified. 5 and AL = Alanine. 6 and TY = Tyrosine. 7 and V = Valine. 8 = Unidentified. 9 and I-L = Iso-leucine. P = Phenylalanine. S = Plasma Sample.

TABLE III

THE ESTIMATED CONCENTRATION OF FREE AMINO ACIDS IN NORMAL PLASMA AND IN PLASMA OF CHICKS INFECTED WITH THE PARASITE, EIMERIA TENELLA

Amino Acids	Area of Spots*		Conc. (mg. 100 ml.)
1. Lysine	(a)**	93	6.95
	(b)***	86	6.15
2. Aspartic acid	(a)	73	5.95
	(b)	71	5.85
3. Glutamic acid	(a)	103	7.9
	(b)	86	5.8
4. Unidentified			
5. Alanine	(a)	77	5.9
	(b)	71	5.25
6. Tyrosine	(a)	29	Trace
	(b)	26	Trace
7. Valine	(a)	105	8.2
	(b)	96	7.5
8. Unidentified			
9. Iso-leucine	(a)	92	7.8
	(b)	87	7.35

*Area of spots = the number of squares outlines on graph paper
**(a) = plasma of normal chicks
***(b) = plasma of infected chicks



NORMAL INFECTED

Fig. 2 Fig. 3

Fig. 2-3. Chromatogram of blood plasma of normal and infected chicks. Glutamic acid (#3) and the valine (#7) seem markedly reduced.

The unidentified spots on the chromatograms were in positions #4 and #8. The spot in position #4 was a bright orange color whose position seemed to fluctuate between glutamic acid (#3) and alanine (#5). Although the spot in position #8 had the characteristic purple color, its intensity was very faint and its R_f value did not correspond with the R_f values of any of the amino acids tested.

A two dimensional chromatogram of normal plasma is shown in Fig. 4 and the chamber in which it was run is shown in Fig. 5.

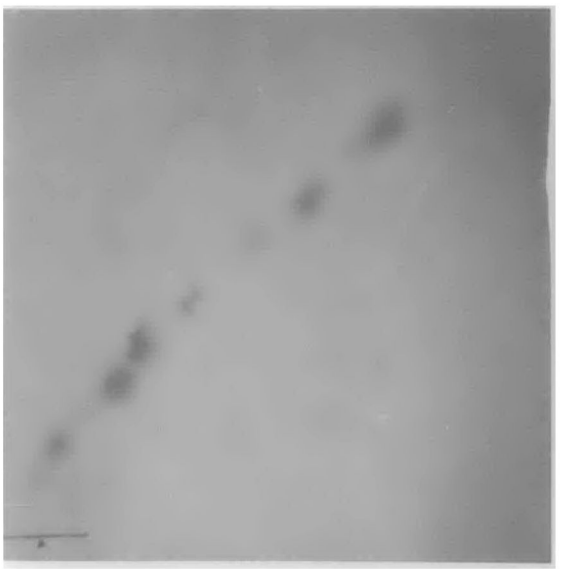


Fig. 4. Two-dimensional chromatogram of blood plasma of normal chicks.

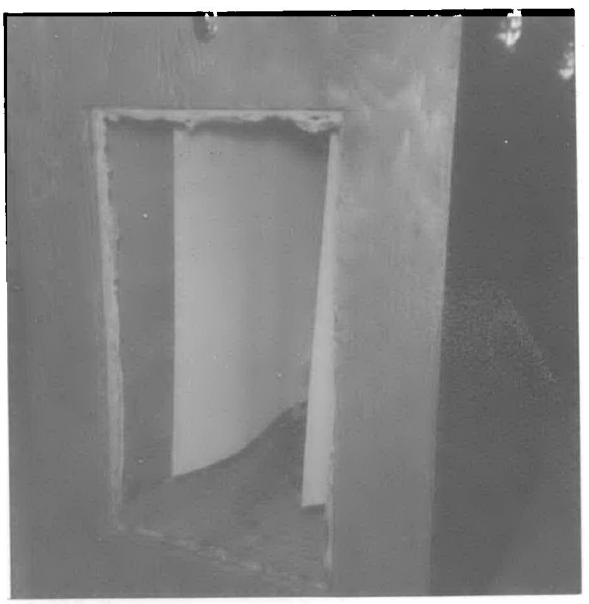


Fig. 5. Chamber used for descending chromatography

CHAPTER V

DISCUSSION

Although many investigators have utilized paper chromatographic techniques in making qualitative determinations of free amino acids in animal tissues, few have used blood as a site for inquiry. Possibly, because of the technical difficulties involved, other techniques have seemed more attractive. Still fewer, if any, have utilized the technique for blood analysis with respect to the coccidian infection.

Walker (1952) found several amino acids in the blood and body tissues of the rat and cow. Among those found in the blood were aspartic acid, glycine, tyrosine, arginine, leucine , lysine, glutamic acid, proline, and valine. Histidine was present in cow plasma but absent in the plasma of rats. Aspartic acid was always present but in small amounts.

Gordon and Nardi (1954), using the paper chromatographic technique, found isoleucine, proline, valine, alanine, serine,threonine, glycine, glutamic acid, cysteine, and aspartic acid in human blood plasma. They also found 6 other ninhydrin positive substances that were not identified.

The results of this investigation reveal that some of the same free amino acids found in the plasma of the rat, cow and humans were also found in plasma of chickens. Although only 7 free amino acids were detected in normal and infected chicken plasma, this doesn't mean that other free amino acids were not present. Other free amino acids could have been

present in such low concentration that their presence was not detected. Faults, inherent with this technique, may also account for the failure of more free amino acids being detected.

The apparent decrease in the concentration of the free amino acids in infected plasma is in keeping with Herrick's observation in 1939 that there is a loss in both total and non-protein nitrogen as a result of the infection. The concentration of glutamic acid especially was apparently reduced. A list of the free amino acids detected in the plasma of infected chicks and their estimated concentration is given in Table III.

Although the method of quantitative assay of amino acids on paper chromatograms by Fisher et al. (1948) is adequately accurate, the modified technique used by this writer may best be called semi-quantitative. There were two primary sources of error that could not be avoided. The first source involved outlining the spots on the graph paper. The writer could rely only on visual acuity while Fisher et al. used a commercial reflex copier (Miles Aircraft Co. "Copycat") to make the spots sharp and clear. Attempts at duplicating spots with sharp outlines were made with a Xerox copier, but with little success. The second source of error involved measuring the spot area. Again, the writer used only visual acuity in counting the squares, whereas the spot area was measured by Fisher with a planimeter. Figure 6 consists of a graph that shows a linear relationship between the concentration and the spot area.

There often appeared a small "yellow spot" between spot #1 and #2, which was thought to be an amino acid during the earlier periods of the investigation. A close-up of this spot is shown in Fig. 8. If color alone was considered, this spot might have easily been misinterpreted as proline. Calculating the R_f value for proline, however, ruled out this

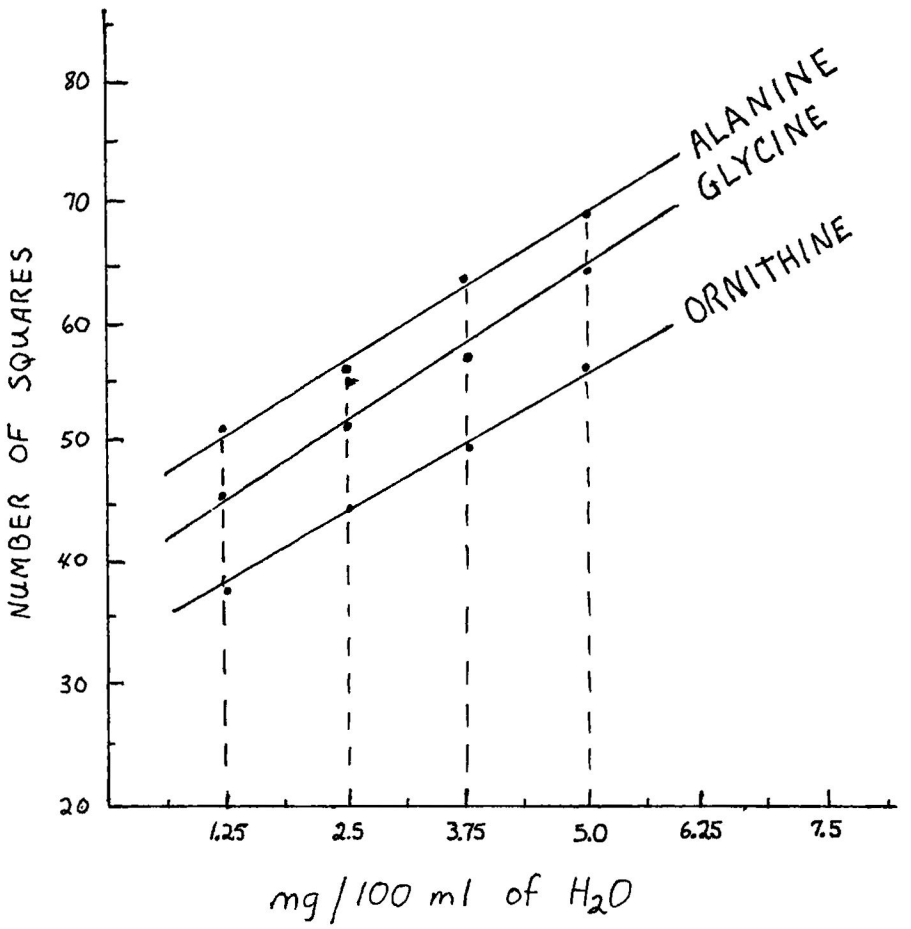


Fig. 6. Graph showing the linear relationship between the concentration of the amino acids and the area of the spots.

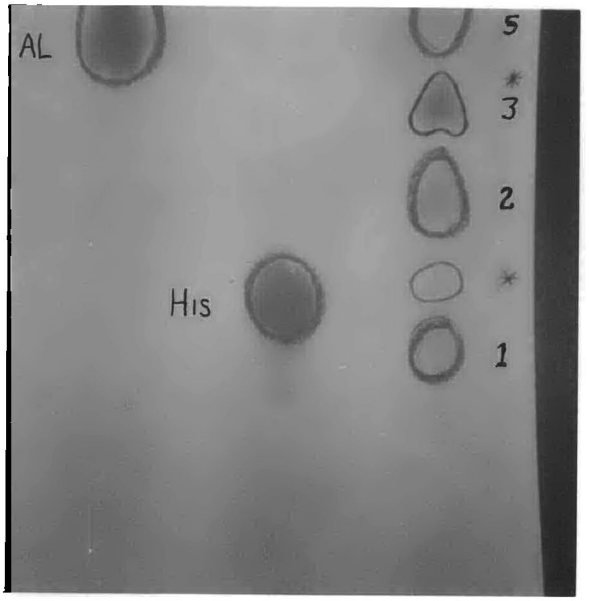


Fig. 7. Seven-hour chromatogram showing the "yellow spot" and the failure of spot #4 to separate. His = Histidine. AL = Alanine.

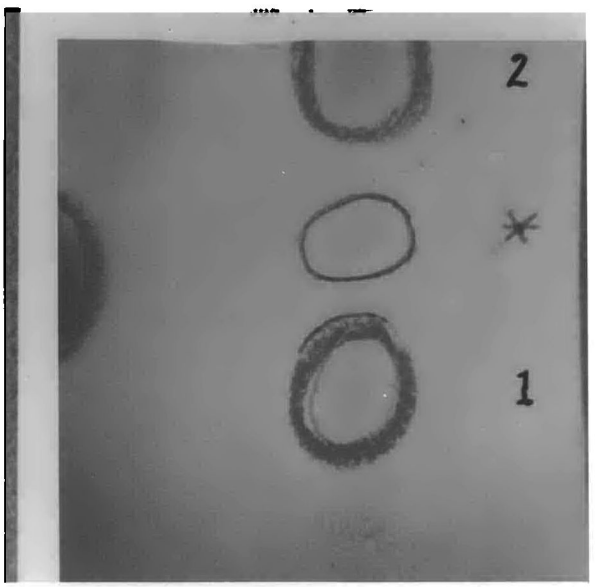


Fig. 8. Close-up of the "yellow spot".

possibility. The possibility of the spot being a blood pigment is worth consideration.

In 1948, Dent observed a phenomenon similar to spot #4. He observed that glutamic acid tended to divide into two spots; one being "redder" than the other (Fig. 7). He suggested that it might be a decomposition product of glutamic acid. Dent, however, used phenol and collidine as his solvents and made two-dimensional chromatograms.

When an iso-leucine sample was chromatographed, there often appeared a smaller fainter spot under a much larger one (Fig. 1). The smaller spot had the same R_f value as the unidentified spot in position #8. This smaller spot may be a decomposition product of iso-leucine or the iso-leucine sample may have contained traces of another unidentified amino acid. Further speculations as to the identity of these spots may not be justified.

CHAPTER VI

SUMMARY AND CONCLUSIONS

1. A paper chromatographic study of the free amino acids in the blood plasma of normal chicks was made and of chicks infected with the coccidian parasite, Eimeria tenella.
2. Seven free amino acids were detected. These were iso-leucine, valine, aspartic acid, tyrosine, glutamic acid, alanine and lysine.
3. Two ninhydrin-positive substances were unidentified.
4. An unidentified "yellow spot" was also detected in some of the chromatograms.
5. The apparent reduction in the concentration of the free amino acids following the coccidian infection, strongly suggests that there is a definite relationship between Eimeria tenella infection and the concentration of free amino acids in blood plasma of White Leghorn chicks.

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